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13. ABSTRACT (Maximum 200 words)

This grant has been renegotiated and the new technical objectives are:

1) To determine whether LPS induces tyrosine phosphorylation of endothelial cell proteins that can be coupled to changes in barrier function.

2) To determine whether this LPS-induced endothelial intercellular gap formation and barrier dysfunction is due to zonula adherens and/or focal adhesion disassembly.

3) To determine which protein substrates are the targets of LPS-induced tyrosine phosphorylation.

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FOREWORD

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Simeon E Goldman 6/30/95

PI - Signature Date

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INTRODUCTION:

Endotoxin or bacterial lipopolysaccharide (LPS) is a component of the Gram-negative bacterial envelope that can induce septic shock in the host. LPS can perturb numerous host defense mechanisms which can be turned against the host itself. During Gram-negative bacteremia and its attendant endotoxemia, one key host target tissue for intravascular LPS is the endothelial cell (EC). We previously have demonstrated that LPS induces EC actin depolymerization and intercellular gap formation permitting extravasation of macromolecules through this paracellular pathway (Goldblum et al; *J. Cell Physiol.* 157:13-23, 1993).

Previous studies have demonstrated that LPS can activate multiple signal transduction pathways. Most of these reports have focused on cells of monocyte/macrophage lineage. There is evidence that LPS induces tyrosine phosphorylation of isoforms of the mitrogen-activated protein (MAP) kinases in the macrophage. Tyrosine phosphorylation regulates the state of assembly of cell-cell adherens junctions, the zonula adherens. We hypothesized that LPS induces tyrosine phosphorylation of EC proteins and that this protein tyrosine phosphorylation event could be coupled to LPS-induced changes in endothelial barrier function.

BODY

Methods:

LPS Preparation: Purified native LPS phenol-extracted from *Escherichia coli* serotype 0111:B4 (Sigma) was dissolved in phosphate-buffered saline (PBS) at 1mg/ml and stored at -20°C. For experiments, the LPS stock solution was further dissolved into supplemented tissue culture media as described below.

Endothelial Cell (EC) Culture: Bovine pulmonary EC were obtained from the American Tissue Culture Collection (Rockville, MD). EC were grown at 37°C under 5% CO_2 in DMEM enriched with 20% heat-inactivated (56°C, 30min) fetal bovine serum (FBS) (Hyclone Laboratories, Inc.; Logan, UT), L-glutamine 4mM, nonessential amino acids, and vitamins in the presence of penicillin (50u/ml) and streptomycin (50μg/ml) (Sigma). EC were washed and gently detached with a brief (1-2min) trypsin (0.5mg/ml) (Sigma) exposure with gentle agitation followed immediately by neutralization with FBS-containing medium. EC were counted and suspended in medium for immediate seeding of barrier function assay chambers (see below) (4×10^5 cells/ml) or 100mm tissue culture (7.7×10^4 cells/ml). Cultures were determined to be endothelial by uniform morphology and by quantitative determination of angiotensin-converting enzyme activity with commercially available ^{3}H-benzoyl-Phe-Ala-Pro substrate (Ventrex Laboratories, Inc., Portland, Maine 04103).

Immunoblotting for EC Phosphotyrosine: Postconfluent EC monolayers cultured in 100mm dishes were exposed to LPS or media alone in the presence or absence of tyrosine kinase or phosphatase inhibitors for increasing exposure times. The cells

were then lysed with ice cold lysis buffer containing 50mM Tris-HCL, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150mM NaCl, 1mM EGTA, 1mM PMSF, 1mg/ml leupeptin, 1mg/ml pepstatin, 1mg/ml aprotinin, 100mg/ml DNAse, 1mM Na_3VO_4, 1mM NaF, 10mM pyrophosphate, 500μM paranitrophenol, 1mM phenynlarsine oxide (PAO). The lysates were harvested, centrifuged, (14000 rpm x 10min) and the supernatants collected. In selected experiments where cell lysates were separated into Triton X-100 insoluble and soluble fractions, cells were lysed with ice cold lysis buffer containing 2% Triton X-100, 50mM Tris-HC1, pH 8.0, 1mM PMSF, 10μg/ml leupeptin, 1mM vanadate, 10mM pyrophosphate, 1mM DTT, 500μM PNP, 1mM EDTA, 1mM PAO. The lysates were centrifuged (8000 rpm x 10min), the supernatants (soluble fraction) removed and stored at -80°C. The pellets (insoluble fraction) were brought up in 62.5mM Tris-HCl, pH 6.8, 2.5% SDS, 1% 2-beta-mercaptoethanol, treated with a nuclease solution (1mg/ml DNAse I, 500μg/ml RNAse A, 50mM $MgCl_2$, 50mM Tris-HC1, pH 7.0) for 0.5h on ice, and then acetone precipitated. The precipitate was redissolved by boiling in 62.5mM Tris-HCl, pH 6.8, 2.5% SDS, 10% glycerol. All samples were assayed for protein concentration with a standard Bio-Rad DC Protein Assay (Bio-Rad Chem Div, Richmond, CA). The samples were loaded onto a 8-16% gradient SDS-PAGE gel, electrophoresed and transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane blot was blocked (5% dry milk in PBS x 2h), rinsed, and incubated with biotinylated 4G10 anti-phosphotyrosine monoclonal antibody (0.2μg/ml) (Upstate Biotechnology Inc., Lake Placid, NY) for 1h. The membrane was then washed twice with PBS and incubated with HRP-conjugated

streptavidin for 0.5h. The membrane was then rinsed 5 times in 0.05% Tween-20 in PBS followed by PBS alone x 5. The blot was then developed with enhanced chemiluminescense (Amersham, Arlington Heights, IL) and exposed to X-ray film (DuPont) for increasing exposure times. Autoradiographs were scanned by laser densitometry (Molecular Dynamics, Sunnyvale, CA).

Assay of Transendothelial Albumin Flux: Transendothelial ^{14}C-BSA flux was assayed as previously described (Goldblum et al; *J. Clin. Invest.* 93:692-702, 1994). Polycarbonate filters (13mm diameter, 0.4μm pore size) (Nucleopore, Inc., Pleasanton, CA) were treated with 0.5% acetic acid (50°C, 20min), washed in distilled H_2O and immersed in boiling pig skin gelatin (Fisher Scientific, Pittsburgh, PA) solution (5mg/L distilled H_2O) for 60min. The filters were then dried, glued to polystyrene chemotactic chambers (ADAPS, Inc., Dedham, MA), and gas sterilized with ethylene oxide. These chambers which served as the upper compartment for the assay chambers were inserted into wells of 24-well plates, each well containing 1.5ml media and serving as the lower compartment of the assay chamber. Each upper compartment was seeded with 2 x 10^5 EC in 0.5ml media and cultured for 72h (37°C, 5% CO_2). We used ^{14}C-BSA (Sigma) with a specific activity of 30-39μCi per mg protein as the tracer molecule. The baseline barrier function of each monolayer was determined by applying an equivalent and reproducible amount of ^{14}C-BSA (4,800-6,200dpm/0.5ml) to each upper compartment of 1hr at 37°C, after which 0.5ml from the lower compartment was added to 4.5ml of Optifluor Scintillation fluid (Packard Instruments Co., Downers Grove, IL) and counted in a Tri-carb 1500 Liquid Scintillation Analyzer

(Packard). Only monolayers retaining ≥97% of the ^{14} C-BSA were studied. The monolayers were then exposed to LPS 100ng/ml or medium alone. To determine whether the state of EC protein tyrosine phosphorylation mediates LPS-induced changes in barrier function, experiments were performed in the presence of either tyrosine kinase or phosphatase inhibition. EC monolayers were pretreated with genistein (50μg/ml), sodium orthovanadate (2.5μM) or phenylarsine oxide (0.1μM), 0.5h prior to and throughout the LPS or media exposures. Herbimycin A (1μM) was introduced ~16h prior to and throughout the LPS stimulus. Transfer of ^{14}C-BSA across endothelial monolayers was again assayed.

F-Actin Epifluorescence Microscopy: To maintain EC monolayers under identical experimental conditions to our permeability assay, monolayers were directly stained and visualized on polycarbonate membrane filters as previously described (Goldblum et al; *J. Cell. Physiol.* 157:13-23, 1993). EC grown to confluence on filters were exposed for 6h to LPS 100ng/ml or media alone. Selected LPS-exposed and media control monolayers were incubated with herbimycin A (1μM) for 16h prior to and throughout the 6h study period. The monolayers were fixed (formaldehyde 3.7%, 20min), permeabililized (Triton X-100 0.5% in Hepes buffer, 5min), and stained with the F-actin probe, fluorescein-phalloidin (1.65 x 10^{-7}M, 20min) (Molecular Probes). The filters and their attached monolayers were mounted cell-side up on microscope slides and photographed through a Zeiss Axioskop 20 Microscope equipped for epifluorescence.

RESULTS

LPS-Induced Tyrosine Phosphorylation of EC Proteins: EC were exposed to LPS 100ng/ml or media alone for increasing exposure times (5min to 6h) and lysed in lysing buffer containing Triton X-100 and protease and phosphatase inhibitors. The Triton X-100 insoluble fractions were solubilized, separated by SDS-PAGE (30μg protein/lane) and transferred to PVDF membranes. The blots were blocked, rinsed, and incubated with biotinylated murine monoclonal antiphosphotyrosine IgG $2b_k$ antibody (Upstate Biotech Inc., Lake Placid, NY) followed by HRP-conjugated streptavidin. The blots were developed with enhanced chemiluminescence, exposed to Kodak XAR X-ray film, and the autoradiographs were scanned by laser densitometry.

The Triton X-insoluble fractions from LPS-exposed EC compared to their paired simultaneous media controls (same gel, adjacent lane), were analyzed for phosphotyrosines. In the insoluble fraction from EC exposed to LPS for 1h there was a significantly ($p < 0.01$) increased phosphotyrosine(s) with MW $\approx$95kDa (Figure 1A and B). This phosphoprotein was ~1.8-fold increased compared to the simultaneous media control. This increase in the 95kDa phosphoprotein could not be demonstrated at any other time point (Figure 1A). In Figure 1B, all the EC extracts were harvested from EC exposed to LPS (L) or media (C = control) at 1h as a further check on the significant increments in the 95kDa phosphotyrosine seen after the 1h LPS exposure. The 1h LPS-induced increment was confirmed.

Effects of Tyrosine Kinase and Phosphatase Inhibition on LPS-induced Endothelial Barrier Dysfunction: To determine whether LPS-induced changes in endothelial barrier function were mediated through tyrosine phosphorylation of EC proteins, two structurally dissimilar tyrosine kinase inhibitors, herbimycin A and genistein, were introduced into the barrier function assay. Pretreatment with either genistein or herbimycin A significantly diminished the LPS-induced increments in transendothelial ^{14}C-BSA flux (Figure 2). In fact, the barrier function seen after LPS exposure in the presence of herbimycin A was no different than that seen after the simultaneous media control (0.038 $\pm$ 0.003 *vs* 0.039 $\pm$ 0.002 pmol/h). For the specific mean ($\pm$SE) barrier function and *n* for each experimental and control group - see Table 1. Therefore, two structurally dissimilar tyrosine kinase inhibitors, herbimycin A and genistein, each significantly protected against LPS-induced loss of barrier function. This suggested that changes in the state of tyrosine phosphorylation of one or more EC proteins was essential to the LPS effect. Since the state of protein tyrosine phosphorylation is dynamically regulated by both "kinases" and "phosphatases", we reasoned that inhibition of tyrosine phosphatases would protect against dephosphorylation, i.e. prolong phosphorylation, and enhance the LPS effect. Postconfluent EC monolayers cultured on filters mounted in barrier function assay chambers were treated with one of two tyrosine phosphatase inhibitiors, sodium orthovanadate (2.5μM) or phenylarsine oxide (PAO) (0.1μM), each for 0.5h prior to and throughout the 6h LPS (100ng/ml) exposure. Simultaneous controls with vanadate, PAO, or medium alone, each were performed. At 6h, transendothelial ^{14}C-

BSA flux was again assayed. Pretreatment with either vanadate or PAO significantly enhanced the LPS-induced increments in transendothelial ^{14}C-BSA flux (Figure 3). For the specific mean ($\pm$SE) barrier function and *n* for each experimental and control group - see Table 2.

On the basis of previous reports that tyrosine kinase inhibition with tyrphostins protect *vs* LPS-induced lethality in mice, we tested tyrphostin AG-126 in our *in vitro* endothelial barrier function assay system. First, we demonstrated that 9h-exposures to tyrphostin AG-126 over a concentration range of 1-100μM failed to significantly increase ^{14}C-BSA flux compared the simultaneous media control (Figure 4). Second, we found that tyrphostin-AG-126 100μM introduced 3h prior to and throughout a 6h LPS exposure (100ng/ml) failed to protect against LPS-induced loss of barrier function (Figure 5). We performed these experiments as a first test as to whether tyrphostin AG-126 might be used for planned *in vivo* experiments in collaboration with Drs. W. Wiesmann and F. Pierce at WRAIR. Our combined data suggest that herbimycin A (or genistein) will be preferable for tyrosine kinase inhibition in LPS-challenged animals.

Effect of Tyrosine Kinase Inhibition on LPS-Induced Intercellular Gap Formation: F-actin probed endothelial monolayers exposed to LPS 100ng/ml or medium alone for 1.0hr were photographed through an epifluorescence microscope (Figure 6). All medium control and herbimycin A-treated monolayers contained continuous transcytoplasmic actin filaments and tight cell-to-cell apposition without intercellular gaps (Figure 6A and B). After LPS exposure, isolated ellipsoid disruptions within the

F-actin lattice could be seen (Figure 6C). These discrete disruptions occurred exclusively at the cell-to-cell interface. Prior treatment with the tyrosine kinase inhibitor, herbimycin A, dramatically protected against LPS-induced intercellular gap formation (Figure 6D).

Effect of Tyrosine Kinase Inhibition on the Ability of Multiple Agonists to Compromise Endothelial Barrier Function: Monolayers cultured in assay chambers that exhibited functional integrity on the basis of baseline barrier function were exposed to media, LPS 100ng/ml, TNFα 1000μ/ml, SPARC 15μg/ml, or thrombospondin (TSP) 15μg/ml, all for 6h. Some of these monolayers were also pretreated with herbimycin A 1μM for 16h prior to and throughout the 6h study period. Prior tyrosine kinase inhibition protected against LPS-, TNFα-, SPARC-, and TSP-induced loss of barrier function (Figure 7).

CONCLUSIONS:

1. LPS induces tyrosine phosphorylation of an EC protein(s). Preliminary data suggests that the phosphoprotein has a MW≈95kDa, resides within the cytoskeletal (Triton X-100-insoluble) fraction, and appears after a 1h LPS exposure.
2. Tyrosine kinase inhibition protects against LPS-induced loss of endothelial barrier function.
3. Tyrosine phosphatase inhibition exascerbates the LPS-induced loss of endothelial barrier function.
4. Tyrophostin AG-126, a more specific tyrosine kinase inhibitor, did not intrinsically influence barrier function nor did it protect against the LPS effect.
5. Tyrosine kinase inhibition protected against LPS-induced intercellular gap formation.
6. Tyrosine kinase inhibition protected against the ability of multiple agonists to compromise endothelial barrier function (suggesting a final common pathway).

APPENDIX

A. Tables 1-2

B. Figures 1-7

The Effects of Tyrosine Kinase Inhibitors on LPS Induced Permeability in CPAE Cells

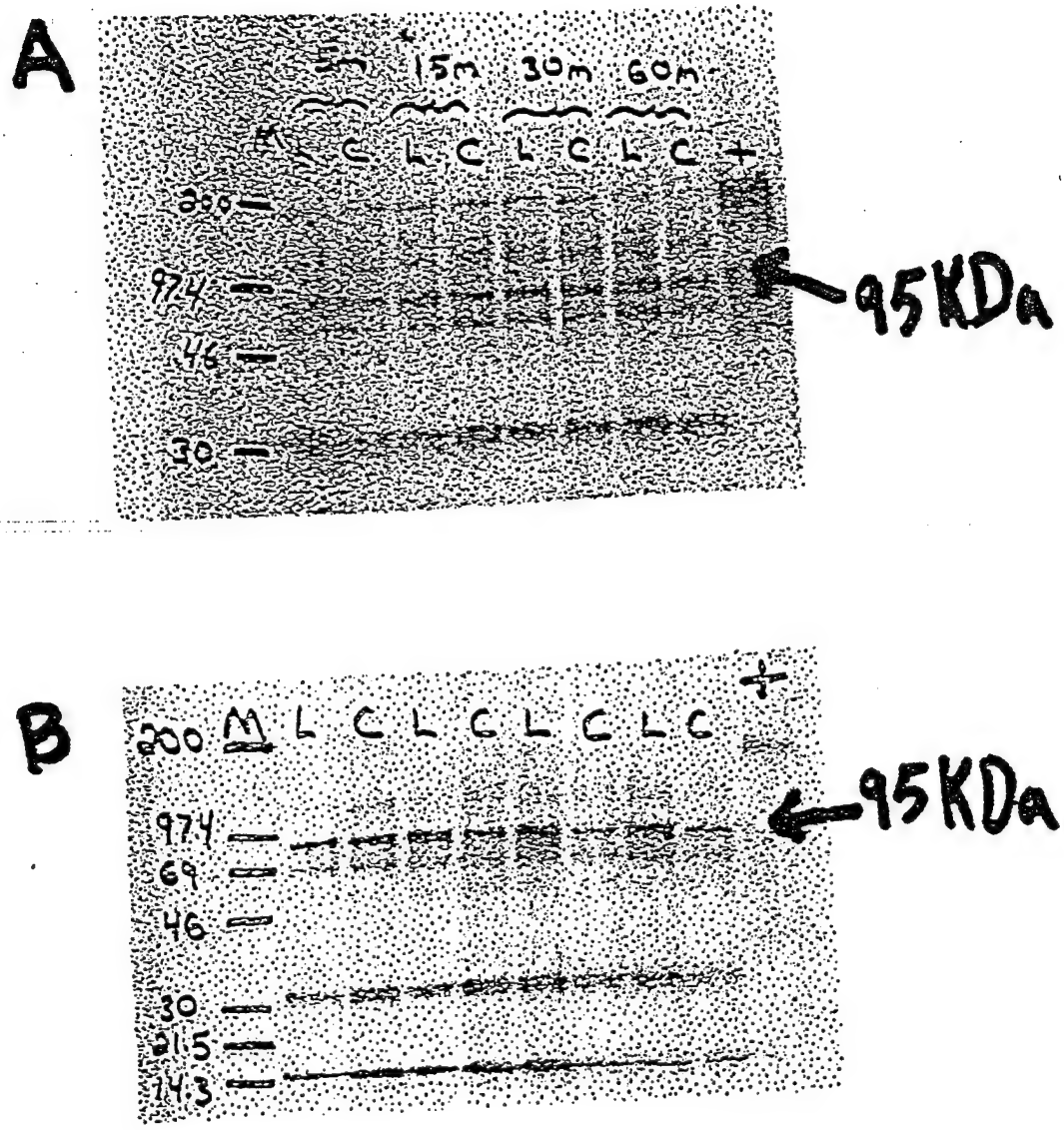
Condition	pMole	n	SEM
baseline	0.012457	38	0.00049
media control	0.039	9	0.0036
LPS (100 ng/ml)	0.0933	8	0.0069
Genistein (50 ug/ml)	0.0261	9	0.0032
Genistein + LPS	0.0515	12	0.0045
baseline	0.011096	55	0.00055
media control	0.039	17	0.002263
LPS (100 ng/ml)	0.077	15	0.006236
Herbimycin A (1 uM)	0.021	8	0.001972
Herbimycin A + LPS	0.038	15	0.002779

Table 1

The Effect of Phosphatase Inhibitors on LPS Induced Permeability in CPAE Cells

Condition	pMole	n	SEM
baseline	0.0097	53	0.0005
media control	0.0406	11	0.002
PAO (0.1 uM)	0.0358	11	0.002
LPS (10 ng/ml)	0.0886	11	0.005
PAO + LPS	0.1053	20	0.003
baseline	0.0081	26	0.0006
media control	0.0348	6	0.0041
Vanadate (2.5 uM)	0.0352	5	0.0032
LPS (10 ng/ml)	0.077	6	0.0057
Vanadate + LPS	0.108	9	0.0059

Table 2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5m
15m
30m
60m
M C L C L C L C +
200
97.4
46
30
95KDa
B
200 M L C L C L C L C +
97.4
69
46
30
21.5
14.3
95KDa

Figure 1

The Effect of Tyrosine Kinase Inhibitors on LPS Induced Permeability in CPAE

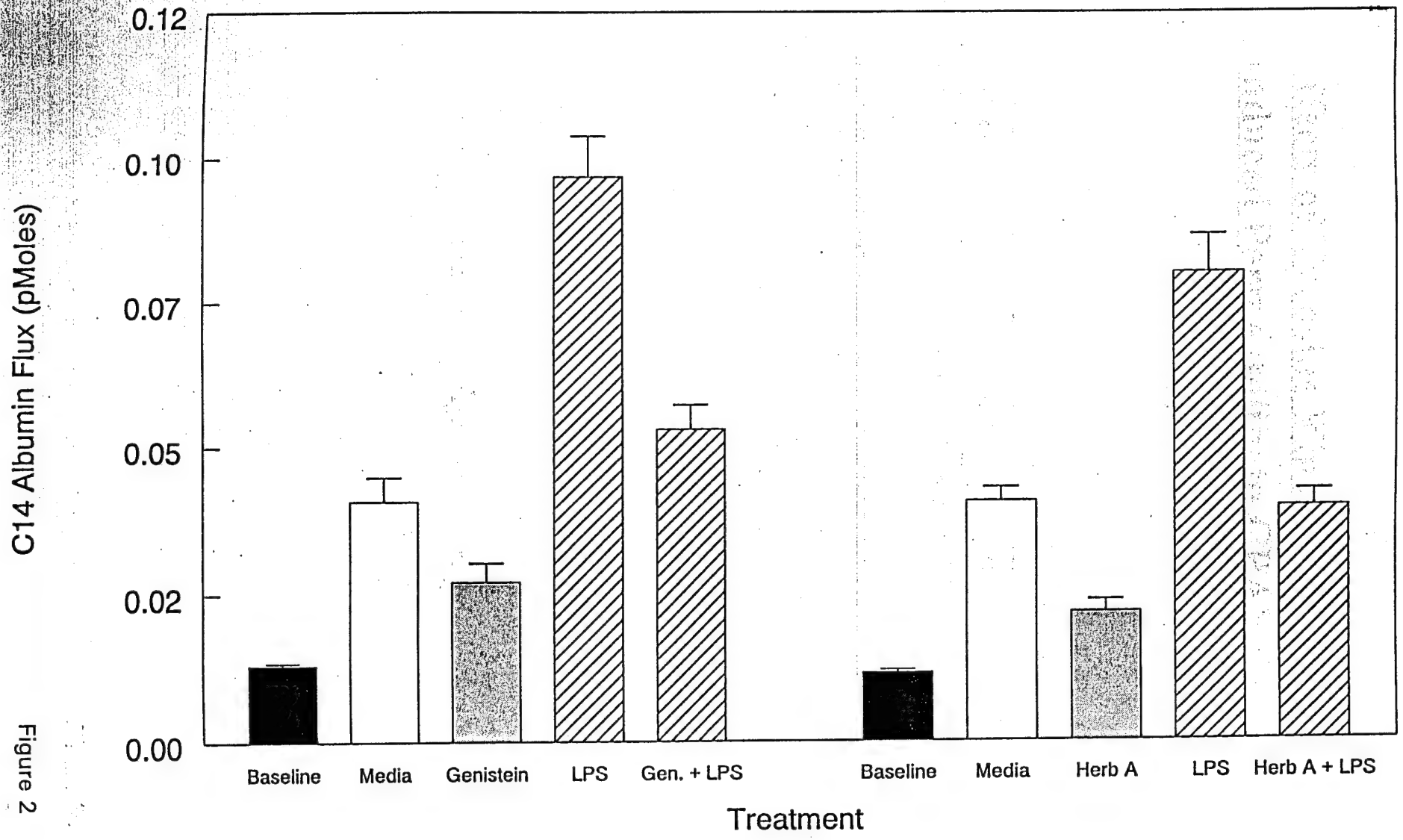


Figure 2

The Effect of Phosphatase Inhibitors on LPS Induced Permeability in CPAE

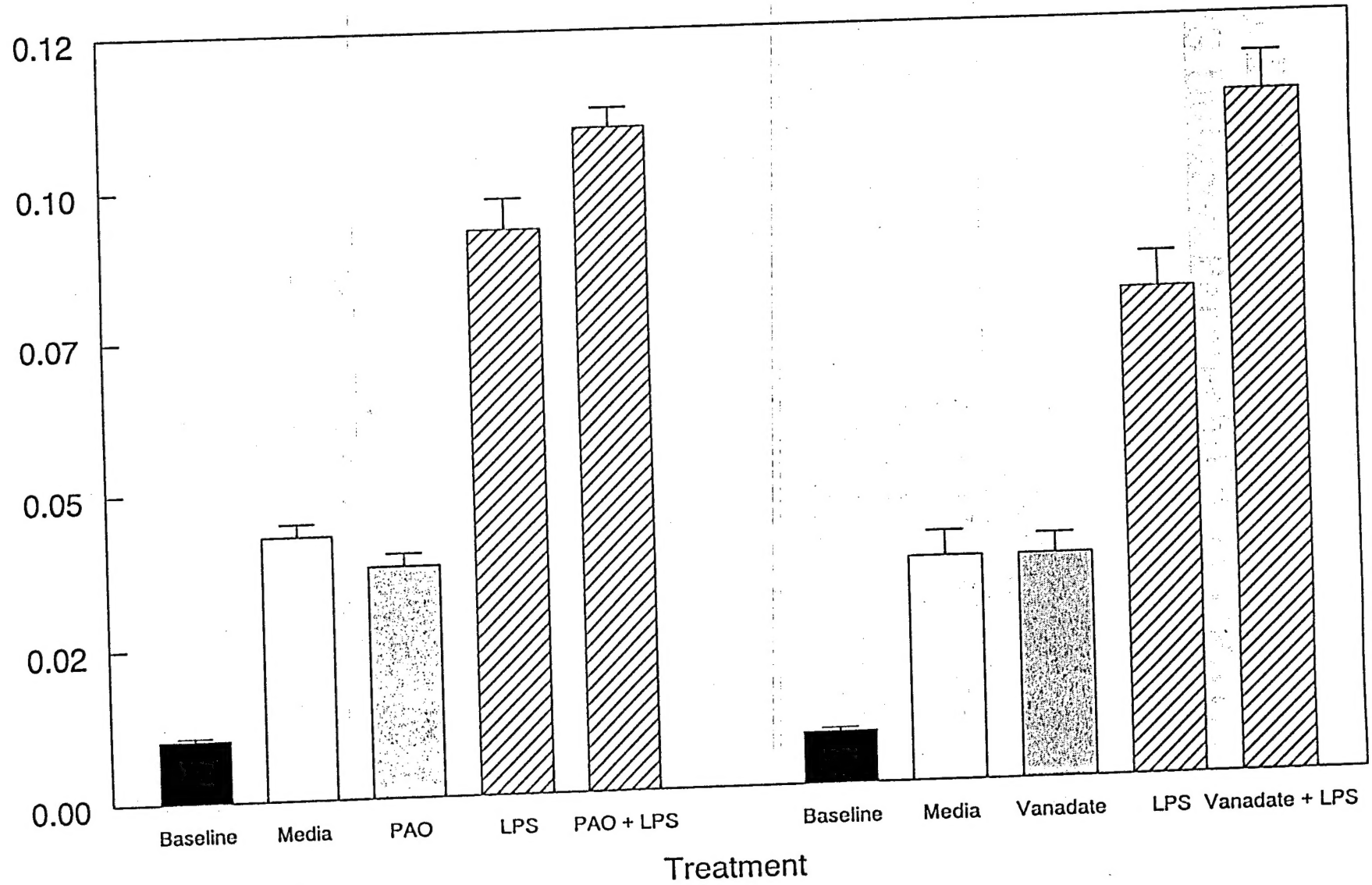
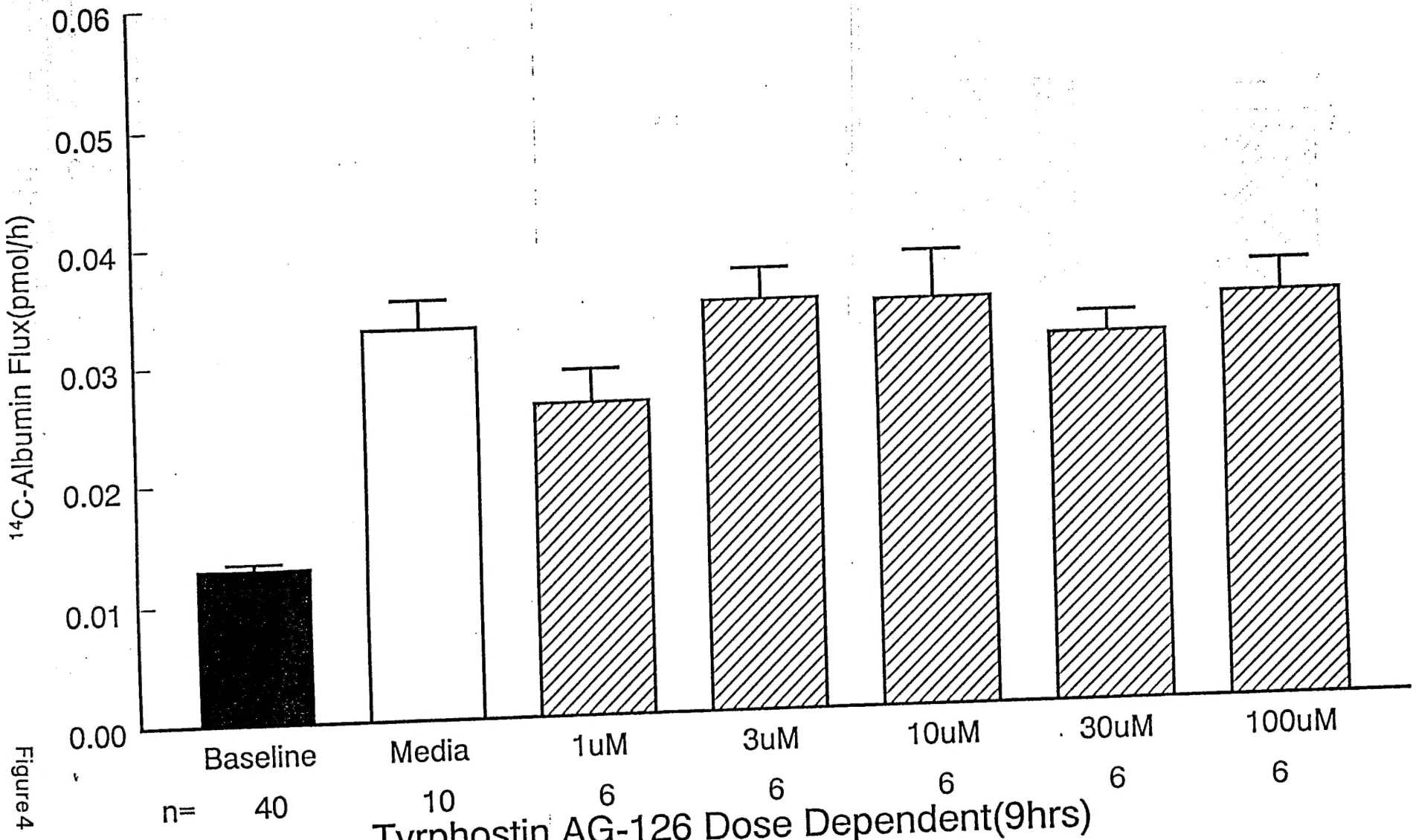


Figure 3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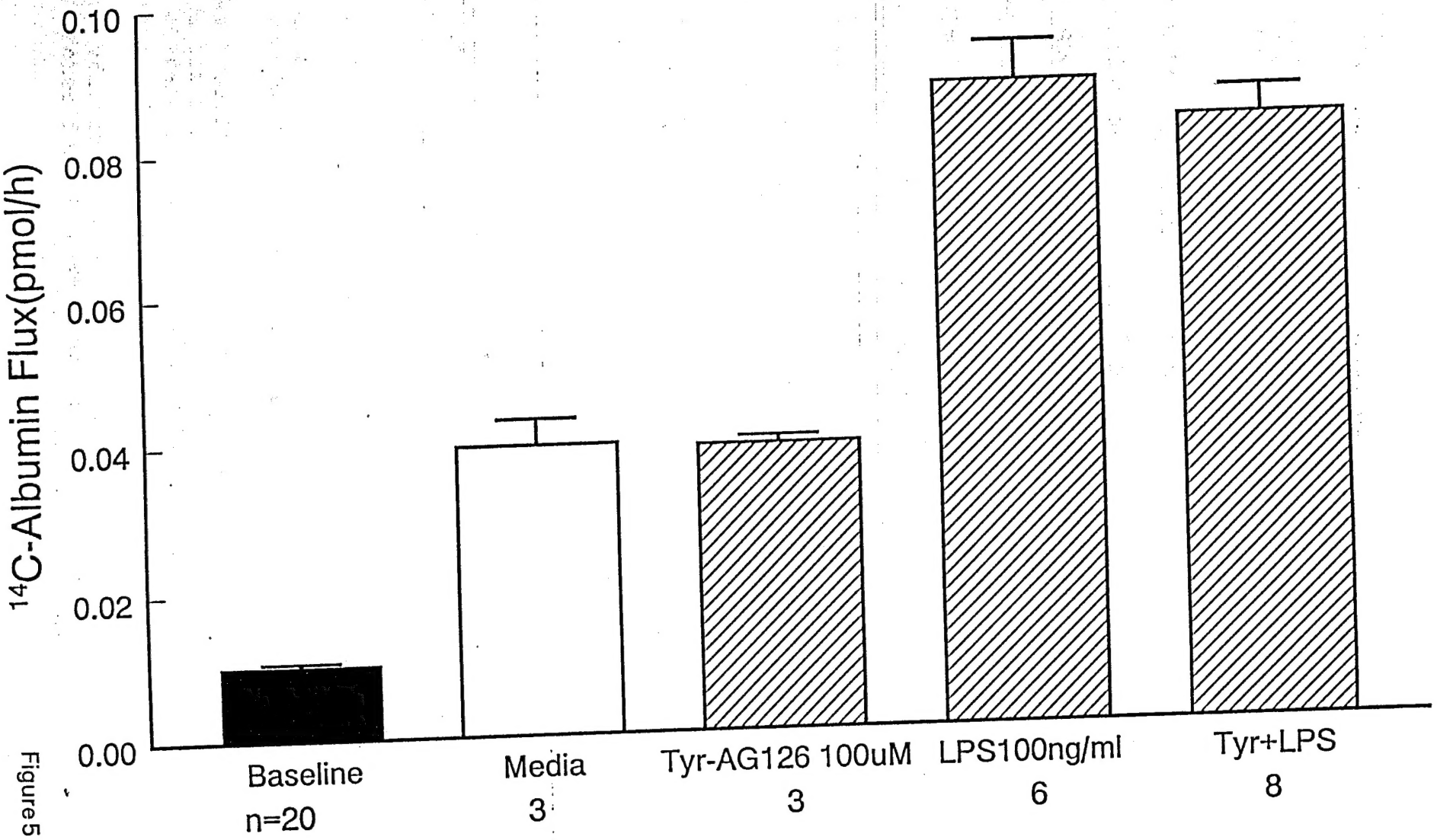
^{14}C-Albumin Flux(pmol/h)
Baseline
Media
1uM
3uM
10uM
30uM
100uM
n=
40
10
6
6
6
6
6
Tyrphostin AG-126 Dose Dependent(9hrs)

Figure4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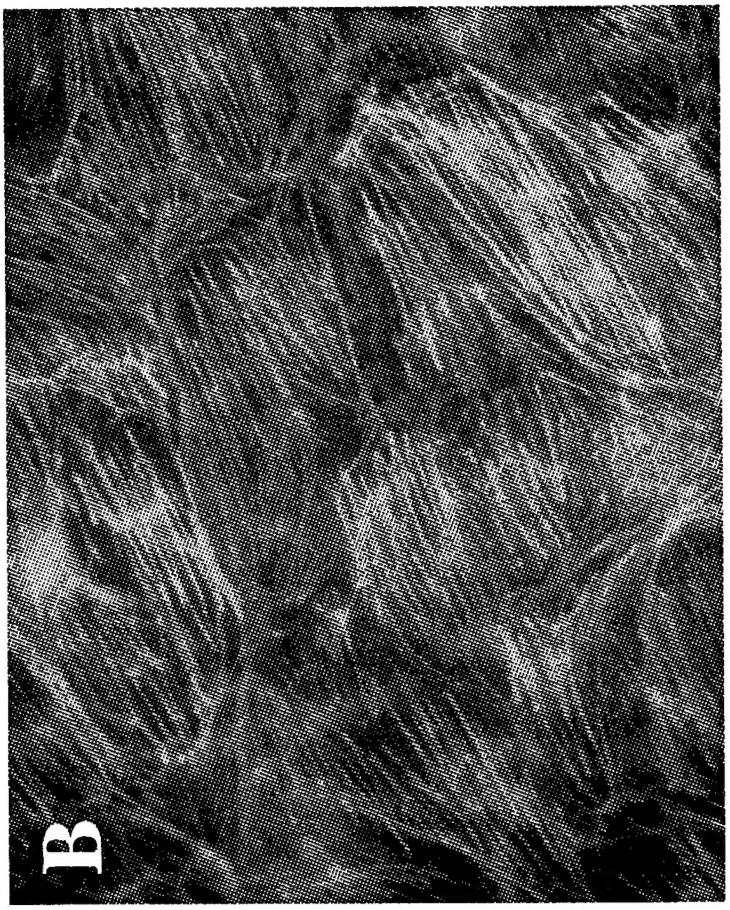
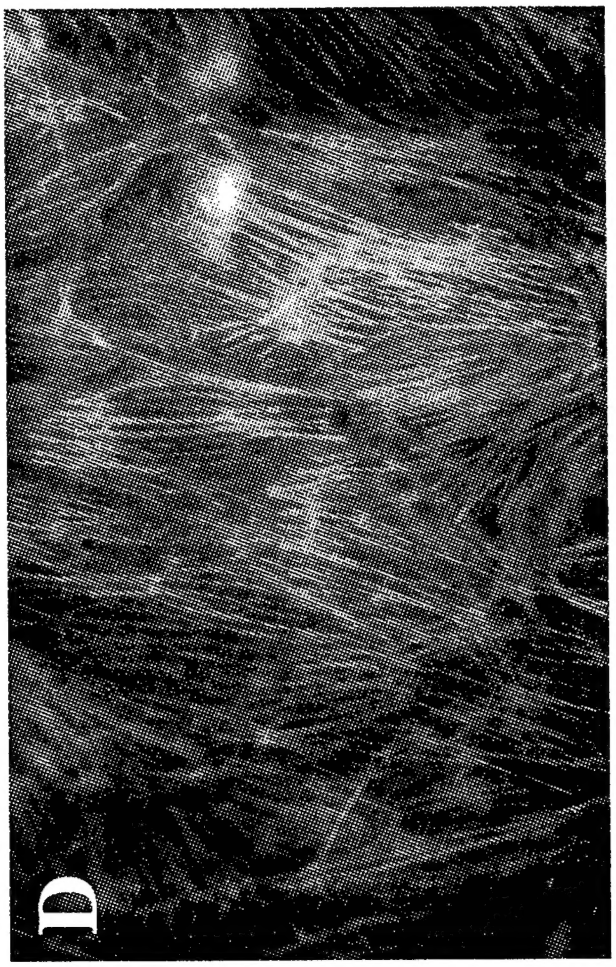
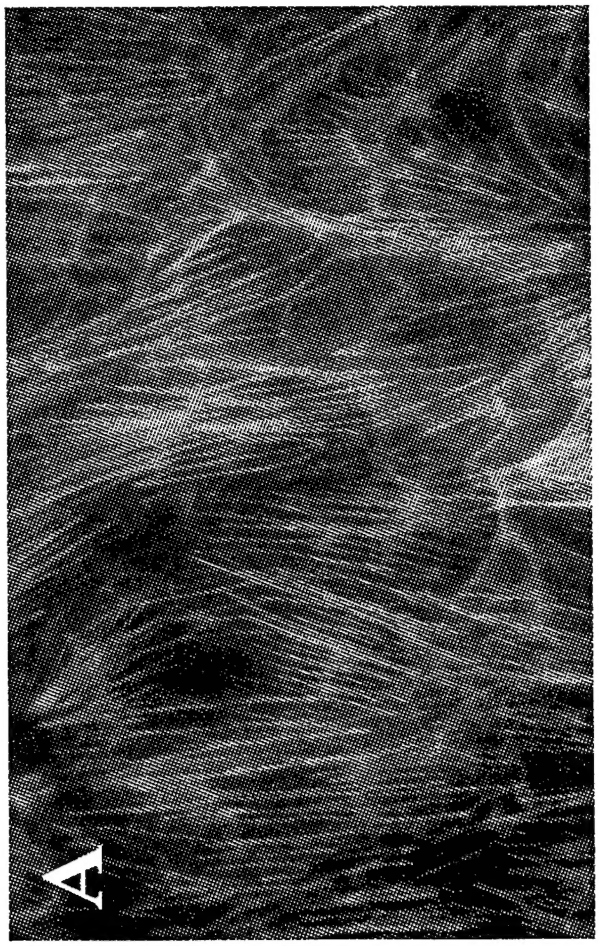
0.06
0.04
0.02
0.00
^{14}C-Albumin Flux(pmol/h)
Baseline
n=20
Media
3
Tyr-AG126 100uM
3
LPS100ng/ml
6
Tyr+LPS
8

Figure5







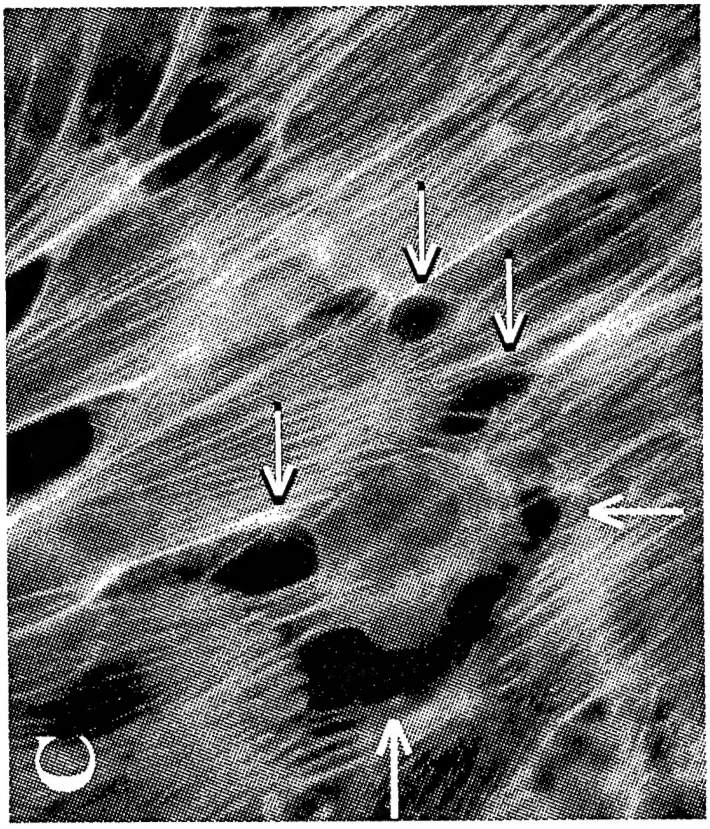


Figure 6